Implant Surfaces

White Paper

Dictating the Short- and Long-term Functional Success of Orthopedic Implants Using an Immuno-optimized Surface

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Implant Surfaces has developed the IntimateBond™ family of **immuno-optimized** surfaces that modulate immune response and preferentially induce target cells to attach directly to implants and grow out from the surface to ensure device fixation and implant performance within the human body.

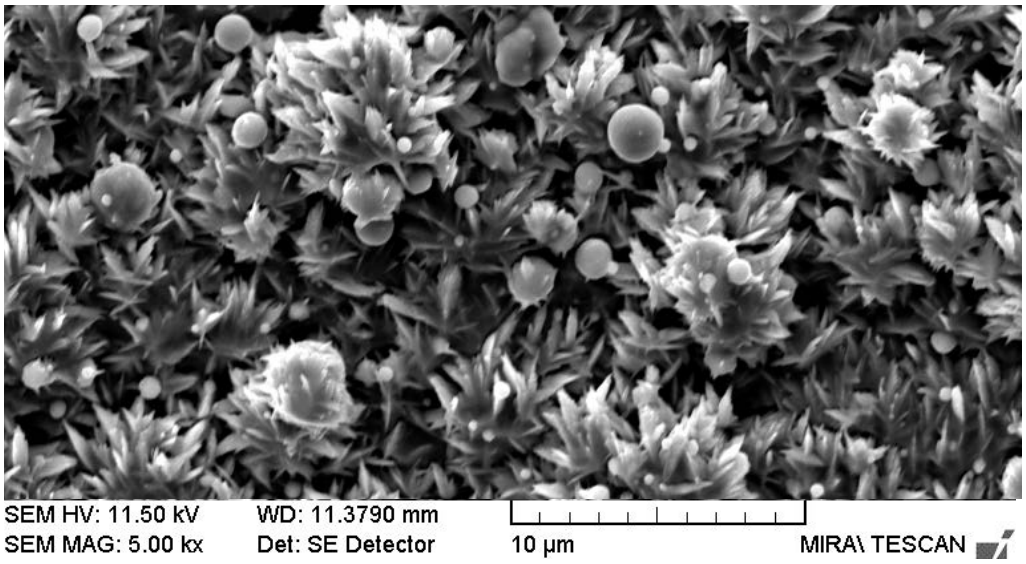

Figure 1. Example of an IntimateBond™ Immuno-optimized cp-Ti surface for cell attachment to an implant.

This paper reviews how the immune system adversely impacts biocompatible implants and how Implant Surfaces uses **immunological modulation design** to develop **immuno-compatible** and **immuno-optimized** surfaces by focusing on the development of a surface for osteoblasts and their attachment to and proliferation on orthopedic implant materials to eliminate or significantly reduce the causes of implant fixation failure.

Immunological modulation design is the process of identifying how the material surface properties affect the body's immune response, for both good and bad. Then systematically identifying modifications to surface properties that:

- improve immuno-compatibility (reducing negative aspects of immune response such as excessive inflammation and macrophage secretion of degrading compounds and the resulting start of chronic fibrotic response), and
- avoid chronic fibrotic response which inevitably leads to implant problems.

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Immuno-compatibility is the ability of a surface to modulate the body's immune response to avoid the negative aspects of that response which routinely compromise implant fixation and performance.

Immuno-optimization is the fabrication of a material surface with the distributions of sub-nano and nanoscale surface elements, topologies and surface charges required to produce a desired result such as attachment and proliferation of a specific cell type.

The result is surfaces that can modulate the cascade of biological events from initial implantation through fixation and thus dictate both the short-term and long-term functional success of an orthopedic implant. This is the same methodology that Implant Surfaces used to identify the different surface properties required by each of several different cell types:

Vascular Endothelials / Mesenchymal Stem Cells (MSC's) / Gingival Fibroblasts / Periodontal Ligament Fibroblasts / Cell attachment prevention surface.

These surfaces for the different cell types share some characteristics, but each has distinct properties critical to triggering the appropriate biological cascade to induce attachment and proliferation of the target cell type.

Implant Surfaces' **immuno-optimized** osteoblast surface improves the success rate for implant-tissue integration by creating a nano-environmental niche that effectively conjoins bone with artificial implant material. It does so by modulating a sequence of events included in the protein adsorption and immunological response that is activated immediately following tissue damage during surgery.

Implant Surfaces' strategy of applying **immuno-optimized** surfaces to conventional orthopedic material is pivotal in governing the cascade of events of osteoimmunomodulation and osteogenesis required to control the regrowth of bone. Further, the penultimate goal is to better close the inherent and often pernicious gap between mature bone and the implant by the attachment and outward growth of osteoblasts from the implant to the bone.

Titanium is inherently more biocompatible with the body than PEEK (polyether ether ketone), but until the **immuno-compatibility** of the specific titanium surface and its structure is well understood and controlled, the 3D-printing of a Ti64 implant or the application of a titanium coating is foregoing the opportunity to ensure maximal functional biological integration of the implant into the body.

There are over 50 technologies for depositing titanium available, each:

- with numerous available deposition equipment designs that each have distinct capabilities, with
- brands that have option and accessory choices, plus
- the availability of multiple techniques or variations available for most of the technologies,
- each with countless settings for each process parameter.

Because of this, it is imperative to understand the immunological reaction required and how each surface performs. Only then can the surface parameters be properly optimized for a specific cell target.

Without an **immunological modulation** designed, **immuno-compatible** and **immuno-optimized** nano- and sub-nano-structure that is tested, confirmed, and produced under unvarying process control, the probability of producing an implant surface that will ensure maximal functional biological integration appears near zero. At least that's what the math shows.

This paper highlights select research that led to the development and choice of what would become the IntimateBond™ Osteoblast surface, now implanted in well over 50,000 surgeries. The skills learned have been applied to several other categories of cell/tissue types that are suitable targets for implant fixation.

Background

The pervasiveness of chronic diseases that require diagnosis and surgical procedures continues to grow each year. Orthopedic injuries and disorders, for example, resulted in approximately 19 million procedures performed in the US in 2022.[1] These orthopedic procedures include spinal surgery, knee reconstruction, trauma fixation, hip replacement, cranio-maxillofacial fixation, shoulder replacement and other joint reconstruction procedures.[2] Devices such as joint replacement implants and spinal stabilization implants (e.g. rods, screws, fixation plates, etc.) are able to restore and reconstruct with the intent of accelerating healing times, providing durability while avoiding implant failure and minimizing secondary surgeries.[3-6] The increasing number of patients undergoing treatment in addition to more durable and compatible biomaterials and design is fueling the demand for innovative diagnostic and medical devices.

Orthopedic device design and new materials are continuously being developed to overcome limitations that exist with current bone tissue regrowth strategies.[7-11] Traditional efforts have been made to induce osteogenic differentiation of mesenchymal stem cells directly by generating biocompatible or biologically "inert" substrate surfaces.[12-16.]

Unfortunately, developers, in the pursuit of creating "inert" surfaces for the sole purpose of mesenchymal stem cell differentiation, have failed to apply strategies that, foremost, address protein adsorption and immune cell response as it pertains to bone dynamics. Work completed in the field of osteoimmunology reveals that normal or well-functioning immune cells are required for healthy physical bone formation and initiation, and response of immune cells begins with protein adsorption to a hydrated material surface.[12, 17-23] As a result, control of protein adsorption and immune cell response is a prerequisite for regulation of **osteoclastogenesis** and **osteogenesis.** Implant Surfaces' bone regeneration surfaces can be fine-tuned to induce favorable osteoimmunomodulatory responses through activation of local immune cells via control of adsorbed protein layers early in the cascade.

Implant Surfaces' **immuno-optimized** surfaces are designed to provide medical device implant manufacturers with a cost-effective and high-performing implant surface that overcomes existing challenges with orthopedic implant failures while providing opportunity for user optimization according to application. Titanium, its alloys and oxides, have been extensively used in the biomedical industry due to their excellent mechanical, tribological, anti-corrosion, biocompatibility and antibacterial properties.[24.] Conventional titanium biointerface surfaces such as electro-polished titanium, however, inhibit tissue-implant bond formation because the surface directly limits advantageous protein- and immune cell-

surface interaction, causing unresolved inflammation followed by fibrotic tissue encapsulation and, ultimately, implant rejection.[18,25-29.]

Implant Surfaces' **immuno-optimized** surfaces harness controlled sub-nano and nanoscale topological roughness, without changing surface chemistry, to control the biological response on a surface. **Immuno-optimized** surface candidates can be designed and implemented to control any number of material-host interactions including selective protein attachment (quantity and conformation), inflammation modulation, bone growth-resorption balance and reduced microbial infection.

The success of Implant Surfaces' **immuno-optimized** titanium surfaces extends from its ability to form a high-surface-energy layer using tunable surface structures. Surface properties, including roughness, structure morphology, crystallinity, charge distribution, wettability, free energy and hydroxylation, can be manipulated by using nano- and sub-nanoscale surface structures and can profoundly affect the type, amount, conformation and rate of protein adsorption.[30] Protein adsorption or provisional matrix formation, the first event in blood/tissue-material interaction, triggers activation of the coagulation cascade, complement system and platelets which subsequently control the immune cell and inflammatory response.[14, 30-31]

Mode of Action

Upon implantation of a material in a human body, water immediately adsorbs to the titanium oxide surface, at which point titanium oxide changes its properties due to the electrolytic nature of the surrounding fluid. The hydroxylated molecules of the surface, in their deprotonated form, bind to ions in the surrounding environment. The presence of ions on the surface, such as calcium and phosphate, increases the rate of serum protein adsorption[33-36] and the formation of ionic coordination complexes with the oxide surface provide protein binding sites.[33, 37-38] Multi-structure ionic coordination binding sites, created by the spatial arrangement of nano-structures on an Implant Surfaces' surface, stabilize proteins adsorbed to the surface, preventing denaturation and deleterious conformational changes which could later affect binding of immune cells via receptor binding.

Provisional matrix formation activates the coagulation cascade, complement system and platelets resulting in the downstream activation of polymorphonuclear (PMN) cells, monocytes and macrophage immune cells residing in affected tissues. PMNs or first responders migrate chemotactically to the site of injury where they adhere to the provisional matrix layer formed on the substrate. During the acute phase of inflammation, PMNs attach to the protein adsorbed surface and begin chemically destroying and phagocytizing debris and microorganisms. Depending on the compatibility of the material surface with the PMNs, an inflammatory response is triggered via release of cellular enzymes and other soluble factors. Contamination can be defined as anything from microorganism to particle or ion debris released from the surface to an incompatible shape or type of implant material. If PMNs are unable to resolve the contamination, they signal via release of soluble factors for backup from other immune cells. At this point, monocytes, macrophages and other lymphocytes chemotactically migrate to the wound site and adhere to the provisional matrix deposited on the material surface.

Monocytes and macrophages play a vital role in simultaneously modulating the foreign body reaction and osteoclastogenesis-osteogenesis. Once a material surface has been recognized by macrophages and their precursor cells, monocytes then adhere, activate, and

continue the wound healing process. At this point, activated macrophages respond to environmental cues such as the properties of an implant surface through a phenomenon called polarization or phenotype differentiation.

Polarized macrophages adopt a classically active M1 phenotype or an alternatively active M2 phenotype, that of which dictates macrophage behavior on the implant surface via release of pro-inflammatory or anti-inflammatory cytokines, respectively. The anti-inflammatory macrophages (M2) are considered to have regulatory activity and are responsible for promoting cell growth and tissue regeneration. M2 macrophages produce several different mediators such as interleukin IL-1Ra, IL-4, IL-10 and arginase-1 ARG and growth factors including vascular endothelial growth factor A (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β). These soluble factors are responsible for supporting migration, homing and differentiation of bone marrow derived-mesenchymal stem cells (bMSCs).[39-44.]

On the other hand, pro-inflammatory macrophages (M1) are wound healing bodies and have microbicidal and tumoricidal activity.[45] M1 macrophages secrete cytokines such as interleukin (IL)-1β, IL-6, IL-8, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF-α) and interferon (IFN-γ).[39-44] These soluble factors are responsible for promoting inflammation and osteoclastogenesis and in dose and time dependent manners, can induce bone formation.[12] Successful implant-tissue integration relies on having a balance between M1, or pro-inflammatory macrophages to clear and clean the wound site, and M2 or anti-inflammatory macrophages to promote wound healing and regeneration.[45]

It has been well documented that when macrophages are dysregulated or are locked into one type of phenotype when on the surface of an implant, they are unable to successfully guide cells, such as bMSCs, at the implant surface.[46-48] Dysregulation prolongs and continues to propagate inflammation, typically when macrophages are trapped in the M1 phenotype, via continual recruitment of macrophages to the surface. An imbalance of macrophage phenotype may occur because of incompatible surface energy/wettability and/or topography, resulting in dysregulation of the immune system and subsequent pathological disorders.

In general, hydrophobic materials increase monocyte adhesion on a surface compared to hydrophilic surfaces resulting in an immune response.[12,49-50] Positively charged surfaces or entities on a surface that are positively charged tend to cause inflammatory reactions more often and more intensely than negatively charged or neutral species.[12,51-52] Smooth titanium surfaces induce M1 or pro-inflammatory macrophages, causing increased expression of pro-inflammatory cytokines. On the other hand, nano scale morphology provided by controlled deposition supports hydrophilic titanium to induce M2 or anti-inflammatory macrophages, causing increased expression of anti-inflammatory cytokines.[12,45-46,53-54] Structured topography on titanium eliciting both M1 and M2 phenotypes reduces the inflammatory response when compared to unmodified titanium.[12,55-56]

Without desirable material surface properties, the wound healing process fails to follow a physiological pathway and instead pursues a pathological course. Cellular and biophysical events can no longer progress through the normal stages of wound healing, including osteogenesis. Newly recruited macrophages, when induced by fusion-inciting cytokines and a surface that promotes such activity, fuse into foreign body giant cells (FBGCs) in a further attempt to phagocytize rejected material.[46]

Ultimately, congregation of FBGCs on the surface damage and obstruct the function of the device via secretion of reactive oxygen species (ROS), degradative enzymes and acid.[57] These macrophages, through continual production of pro-inflammatory soluble factors, trigger and activate fibroblasts through the release of pro-fibrotic cytokines, essentially causing fibroblasts to continuously synthesize and deposit matrix protein on the implant surface which results in material encapsulation.[58] Osteogenesis is severely compromised or fully impaired. The regulation of macrophage behavior or phenotypic expression by controlling surface properties of an implant is vital for influencing the outcome of bone dynamics.

How Implant Surfaces' Immuno-optimized Surfaces Work

Implant Surfaces' **immuno-optimized** surfaces have favorable bio-physicochemical and bio-mechanical properties that can direct the host immune response as well as osteoinduction, osteoconduction and osteointegration to improve implant integration while avoiding pathways that lead to chronic inflammation, foreign body reaction and subsequent device failure. **Immuno-optimized** surfaces discriminately use surface energy and wettability in balance with topography and roughness to control the amount, type and orientation of protein adsorbed to the surface, which plays a pivotal role in the cascade of biological events including cellular adhesion, morphology, and migration of immunological and osteogenic cells. Furthermore, **immuno-optimized** selective surface properties are designed to interface with osteogenic cells mechano-biologically at a multiscale level (macro-, micro- and nano-) in a manner that significantly modulates bone ongrowth and ingrowth.

To exploit some of the performance benefits of ångstrom-scale and nanoscale, Implant Surfaces has figured out how to manipulate the deposition parameters of several different processes to change the structure of the deposited surface and reduce the "size" of what's deposited down to ions, atoms, molecules, and atom clusters. We have not developed the precision of the researchers who are building clusters one atom at a time, but we have developed the ability to control the size distribution, penetration depth and particle energy of the various cp-Titanium "surface components" when we build surfaces.

This work has provided some important capabilities:

- It has led to creation of our 6 **immuno-compatible** surfaces with the surface characteristics required to elicit the adsorption of targeted proteins onto an implant surface, and
- it has provided significantly enhanced ability to construct nano-laminates of materials.

Implant Surfaces' **immuno-compatibility surface design strategy** revolves around creating favorable protein and cellular responses through implementation of charged, hydrophilic surfaces that coexist with a topographical substratum that caters to all cell types involved in wound healing.

Implant Surfaces' negatively charged or neutral hydrophilic surfaces with sub-nano and nano-scale topology, for example, have been found to beneficially regulate protein adsorption, as well as inflammatory and osteogenic gene expression on implant surfaces, compared to those lacking said features.

Clinical Results

The clinical success of osseointegration and ongrowth/ingrowth fixation of bone at the interface of an implant surface depends on the type and degree of bio and immuno-compatibility. The type of implant material such as metal, ceramic, and polymer, though used throughout history for total joint replacement, for example, elicit different patterns of biocompatibility, ranging from "biotolerance" for PMMA and stainless steel to "bioinert" for carbon and titanium-based alloys.[59]

Implant substrates and surfaces that are "bioinert" tend to promote **contact osteogenesis** whereas materials that are biotolerant tend to promote **distance osteogenesis**.[59] In contact osteogenesis, an implant is colonized by osteogenic cells that migrate to the surface from bone, after which point, they begin synthesizing extracellular matrix and de novo bone in an appositional manner. During this process, an interfacial matrix or non-collagenous cement line is secreted directly on the implant surface by the osteogenic cells connecting or bonding new bone tissue formed on the implant surface with old bone tissue.

In distance osteogenesis, the old bone surface supplies a population of osteogenic cells whose deposition of extracellular matrix moves towards the implant. Bone formation during distance osteogenesis does not occur on the surface of the implant but rather moves to surround the implant surface.[59-60] During bone growth, the surface of the implant is constantly being concealed by cells that could detrimentally intervene in the attachment of osteogenic cells, e.g., fibroblasts. Additionally, osteoblast cells become anchored in their secreted extracellular matrix and eventually die. In general, distance osteogenesis leads to poor bone-growth, not only from lack of bone contact but also from stress shielding of the implant and inducing the progression of a gap between the bone and the implant, contributing to possible long-term failure. In a scenario in which distance osteogenesis predominates due to incompatibility of an implant surface, it can be concluded that proximal bone does not ensure attachment of bone tissue.[59-60]

Characterization of Implant Surfaces' Immuno-optimized Surfaces

Surface Characterization

Implant Surfaces' **immuno-optimized** surface modifications can be easily fine-tuned to achieve surfaces that fit any biological application. Implant Surfaces can alter the topography of a surface without changing its chemistry to achieve a desirable host implant reaction from any number of bulk materials, such as titanium, 304 stainless steel, PEEK, 3D-printed Ti64, etc.

Scanning Electron Microscopy

Titanium-based, **immuno-optimized** surfaces with several different topographies and morphologies have been generated by Implant Surfaces to match the target cell surface attachment requirements for nano-topography, and morphology of the surfaces was observed using scanning electron microscopy (SEM).

Figures 2-8 show SEM images of Implant Surface sample surfaces taken following various surface modification protocols. As shown in the **Figures 2-8**, several different types of cp-Titanium structures and orientations, sizes, crystallinity structures and structure distributions have been achieved on several different implant substrates by varying the control parameters of the titanium deposition process. Each engineered surface promotes a different cascade of cells and attachment preferences.

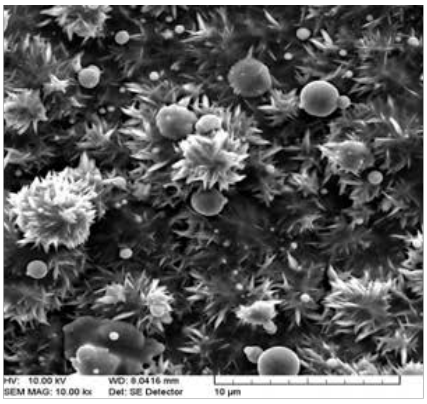


Figure 2. Gingival fibroblast surface

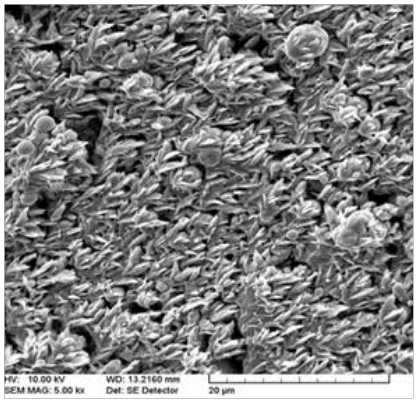


Figure 3. Fibroblast non-adhesion surface

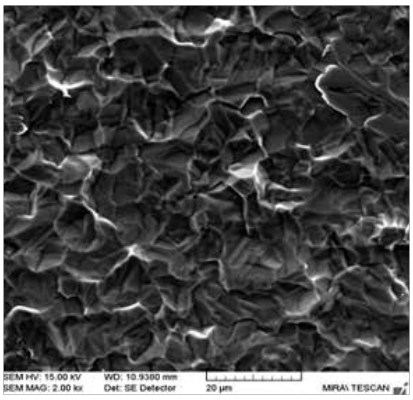


Figure 4. Endothelial cell surface

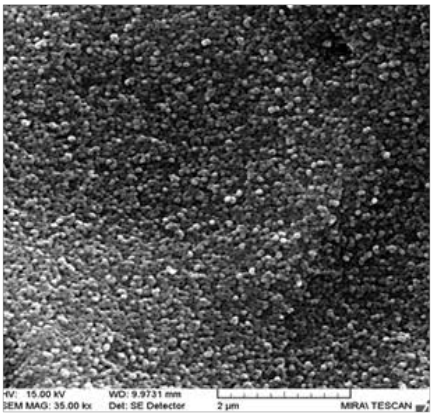


Figure 5. Osteoblast surface

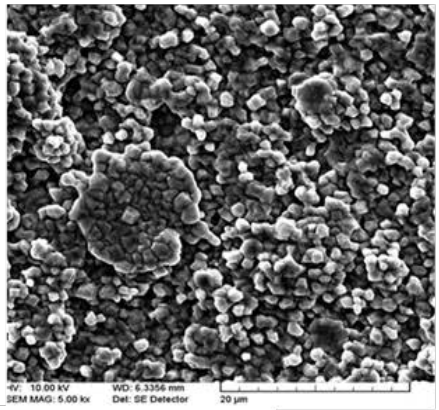


Figure 6. Nerve cell surface

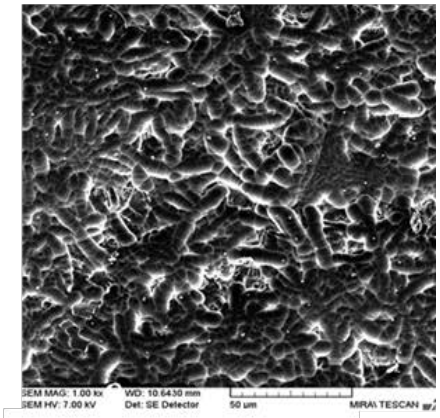


Figure 7. Antimicrobial surface

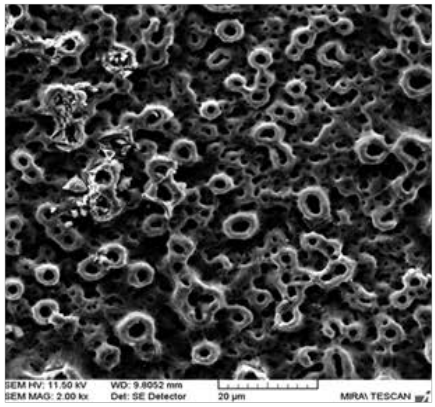
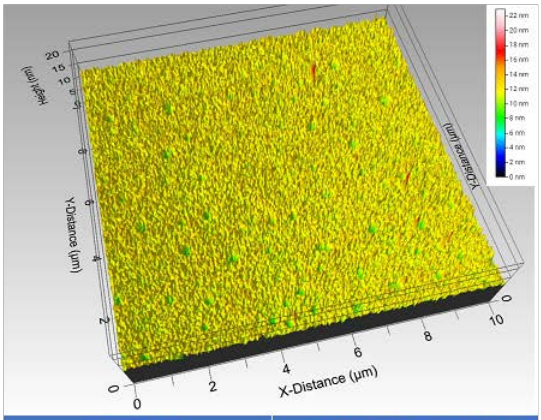


Figure 8. Osteoblast prevention surface

Atomic Force Microscopy (AFM)

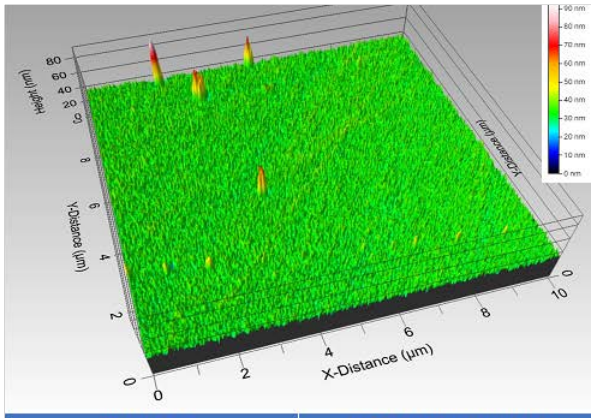
Immuno-optimized surfaces can be fine-tuned to incorporate nanostructures on a surface that produce a range of different RMS roughness values. AFM shows surface roughness potential, measured Root Mean Square (RMS) roughness, between different Implant Surfaces' surface modifications (**Figure 9-12**). The surface illustrated in **Figure 12** has an approximate 45x higher surface roughness compared to the surface generated in **Figure 9**. **Figures 9** and **10** showed a more uniform distribution of nano-roughness, with nano-scale object heights of approximately 10 nm and 35 nm, respectively, compared to **Figures 11** and **12**, with nano-scale object heights that ranged from approximately 100 – 800 nm and 0.2 – 1.8 µm, respectively. A variety of diverse nano-surface diameters (10 – 1000 nm) and roughness values (Ra of 100 nm – 10 micron) have been generated by Implant Surfaces by controlling surface modification process conditions.

Root Mean Square (RMS) Roughness



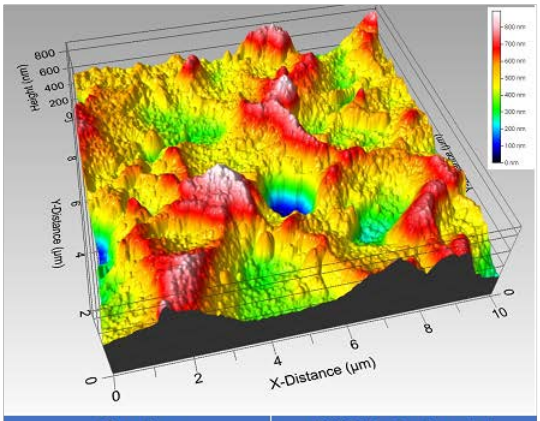
Dimensions	RMS Surface Roughness (nm)
Whole Image	0.933
1µm x 1µm	0.911

Figure 9. RMS Roughness 10 nm



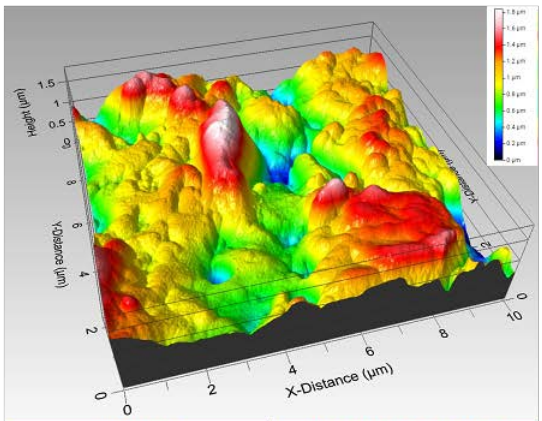
Dimensions	RMS Surface Roughness (nm)
Whole Image	3.47
1µm x 1µm	3.24

Figure 10. RMS Roughness 35 nm



Dimensions	RMS Surface Roughness (nm)
Whole Image	118.7
1µm x 1µm	26.6

Figure 11. RMS Roughness 100-800 nm



Dimensions	RMS Surface Roughness (nm)
Whole Image	290.5
1µm x 1µm	41.4

Figure 12. RMS Roughness 0.2 and 1.2 µm

Contact Angle/Surface Energy

Increasing surface energy and corresponding polar component indicates increasing intrinsic polarity or hydrophilicity and wettability of IntimateBond surfaces. Surface energy data of water and methylene iodide applied to a variety of Implant Surfaces' **immuno-optimized** surfaces on Titanium 6-4 (**Figure 13**) and 316L Stainless Steel (**Figure 14**) was calculated using the Owens-Wendt equation. **Immuno-optimized** surfaces can be fine-tuned to elicit incremental increases in surface energy as well as the polar component of the surface energy based on the type of surface when compared to an untreated material. Each component is finely tuned upon deposition yielding modifications to the measured surface energy.

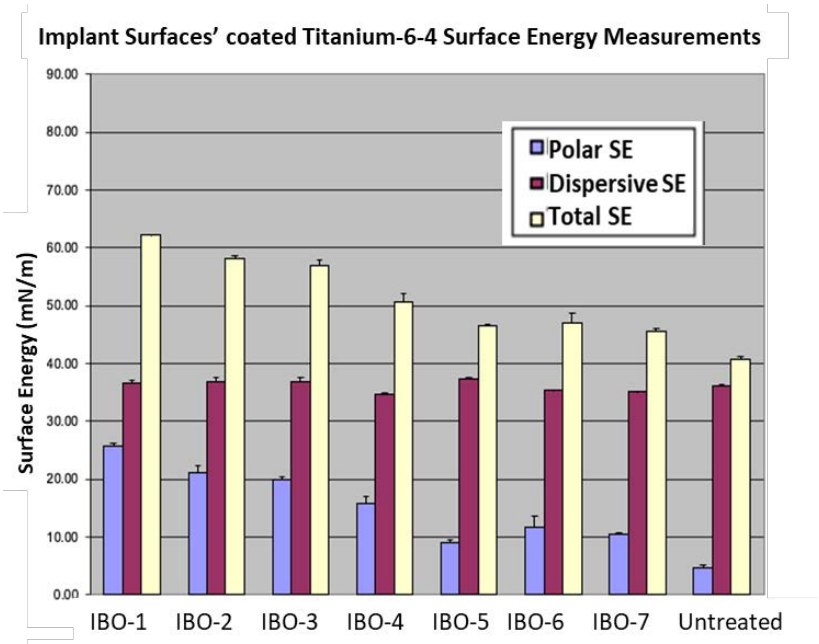


Figure 13. Versions of IntimateBond Osteoblast (IBO) surface energy on Titanium 6-4.

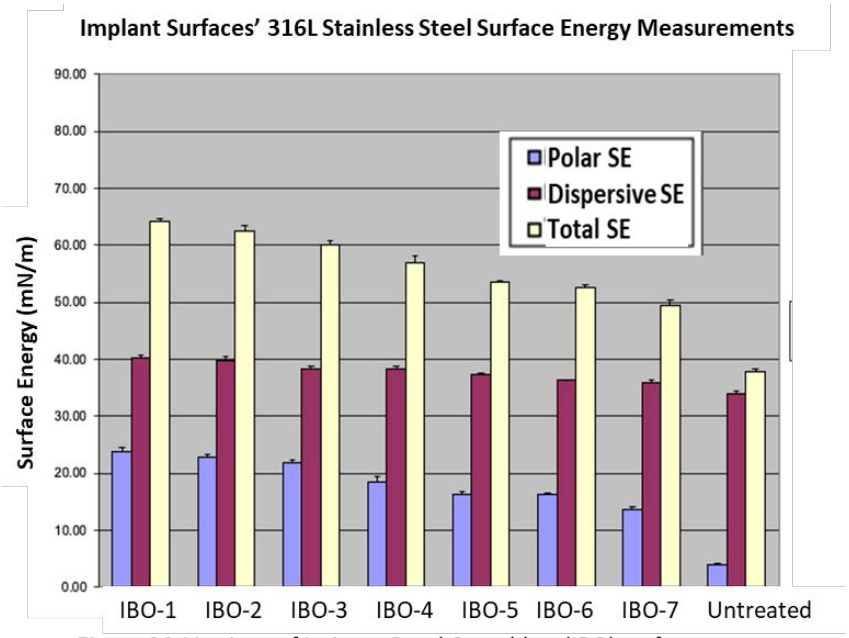


Figure 14. Versions of IntimateBond Osteoblast (IBO) surface energy on Stainless Steel.

Biocompatibility Testing

Protein Adsorption

Selective protein adsorption is one of the first steps in the proliferation of osteoblasts. Results of SEM images showing protein matrix adsorbed to IntimateBond™ Osteoblast surfaces and the distribution of Fibronectin (**Figure 15**) covering the surfaces, including trenches of the topological surface. **Figure 16** illustrates binding to adsorbed fibronectin through chondrocyte cell-surface receptors.

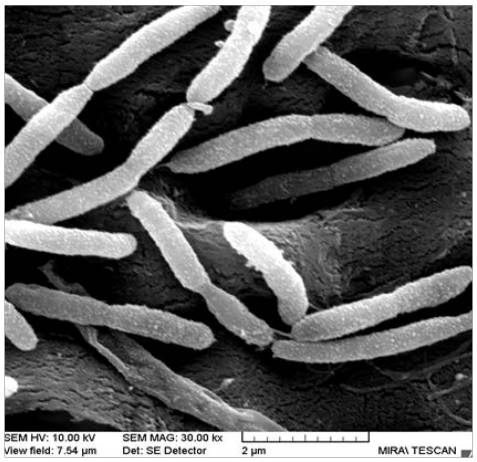


Figure 15. Fibronectin covering surface and trench.

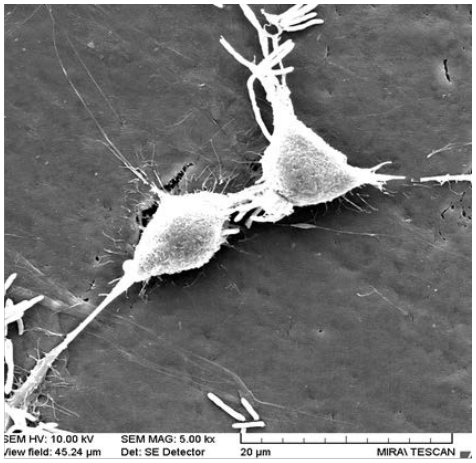


Figure 16. Chondrocyte cell-surface receptors binding to adsorbed fibronectin.

Platelet Adhesion and Activation

An appropriate level of immune response is critical to target osteoblast development. High levels of immune response can be as catastrophic to target cell development as there would be no immune response. It is important to modulate the response for effective development of the target osteoblasts. IntimateBond moderates the immune response by tight control of surface construction. Results of SEM images show significant differences in hemo- and thrombo-compatibility between IntimateBond™ Osteoblast Titanium surface on PEEK (**Figure 18**) versus bare PEEK control based on differences in spatial distribution and morphological changes during platelet activation.

Figure 18, illustrating the **immuno-optimized** surface, shows inactivated platelets or those that are discoidal with no pseudopodia development, yielding a non-thrombogenic surface. In contrast, the control surface (**Figure 17**), shows activated platelets with dendritic (early pseudopodia), spread dendritic (intermediate pseudopodia), spread and fully spread, eliciting early through late-stage activation, respectively, as described by Goodman, yielding a more thrombogenic surface.[61-63]

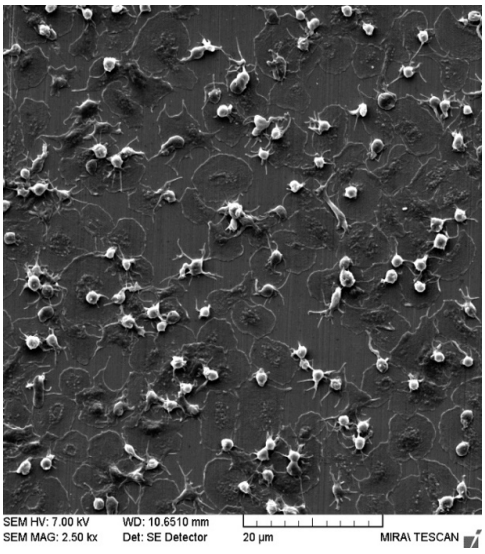


Figure 17. Control - shows platelets with dendritic early through late-stage activation, yielding a much more thrombogenic surface.

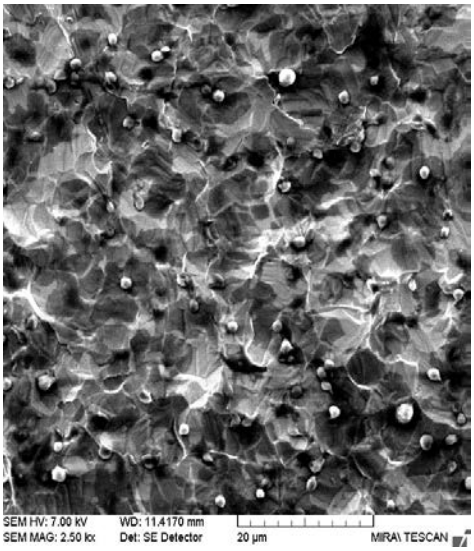


Figure 18. Nano-optimized surface, shows inactivated platelets with no pseudopodia, yielding a non-thrombogenic surface.

Immunological Response

A decrease in cell viability shows that the substrate is cytotoxic to the proliferation of peripheral blood mononuclear cells (PBMC) shown by not stimulating PBMCs for proliferation. **Figure 19** compares Implant Surfaces' IntimateBond™ Osteoblast (IBO) on PEEK versus bare PEEK as control (C). The results show an almost 4.4-fold increase in viability of cells on the IBO surface versus a 2.2-fold decrease in viability on the unmodified surface. In addition, a 1609% increase in cell viability is shown on the IntimateBond™ Osteoblast surface after 96 hours over that on the PEEK surface. This demonstrates that the IntimateBond™ Osteoblast surface is significantly less cytotoxic to PMBC cells than bare PEEK.

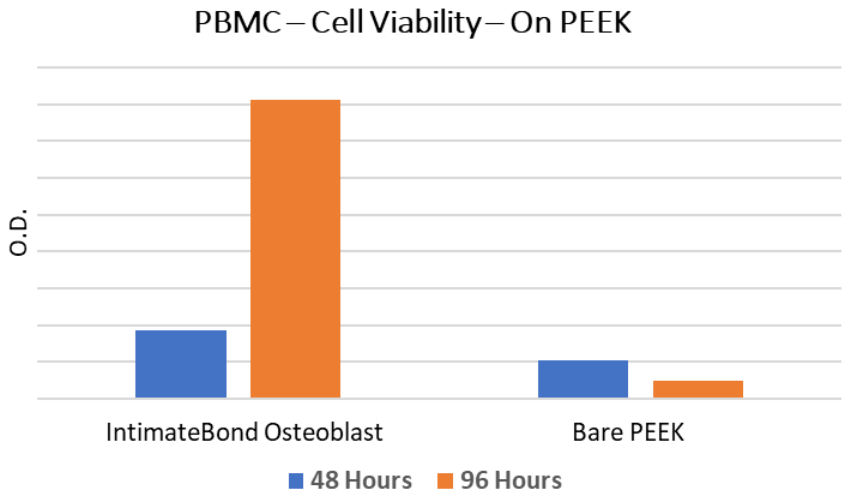


Figure 19. The IBO surface is much less cytotoxic than bare PEEK and supports PBMC

TNF-α is one of the most crucial cytokines for triggering inflammation and an increase in production of pro-inflammatory TNF-α shows that the substrate aggravates the immune response. **Figure 20** evaluates the immune response against the IntimateBond™ Osteoblast surface (IBO) versus bare PEEK surfaces on production of TNF-α cytokine from activated PBMC cells. The significant increase in TNF-α production by PBMCs, at 48 hours and 96 hours, on bare PEEK surfaces, showed that the IntimateBond Osteoblast surface induces far less of an immune response than does bare PEEK. Further, IntimateBond™ Osteoblast may be selectively used to influence the immunological response of the implant system.

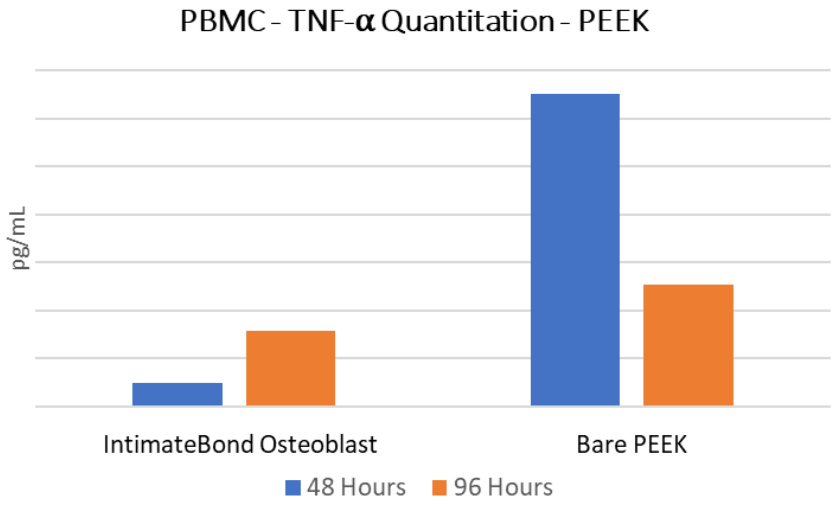


Figure 20. The IntimateBond™ Osteoblast surface is much less cytotoxic than bare PEEK.

Implant Surfaces' **immuno-optimized** surface on cp-Titanium and PEEK showed similar but significantly higher proliferative behavior compared to unmodified cp-Titanium and PEEK. **Figure 21** illustrates the effect of IntimateBond™ Osteoblast on PEEK and applied to cp-Titanium versus uncoated PEEK substrates regarding proliferation of bone marrow derived-mesenchymal stem cells (MSC-bm) expressed in cellular DNA content. IntimateBond™ Osteoblast coated samples showed a rapid increase from day 1 to 3 (635% increase for IS-NC on cp-Titanium and 716% increase for IS-NC on PEEK) and another rapid increase from day 3 to day 7 (308% increase for both IS-NC on cp-Titanium and PEEK), after which point the rate of proliferation drastically slowed and eventually leveled off starting at day 14.

Both unmodified surfaces showed a rapid increase from day 1 to 3 (831% for cp-Titanium and 386% for PEEK). However, proliferation on cp-Titanium drastically slowed starting at day 3 yielding a 173% increase from day 3 to 7 at which point proliferation rate drastically reduced. Unmodified PEEK showed a similar trend leveling off at day 7. Though initially, cp-Titanium and PEEK had high rates of proliferation the maximum quantity of DNA collected from viable cells on unmodified substrates was substantially lower than for both IntimateBond surfaces applied to cp-Titanium and PEEK.

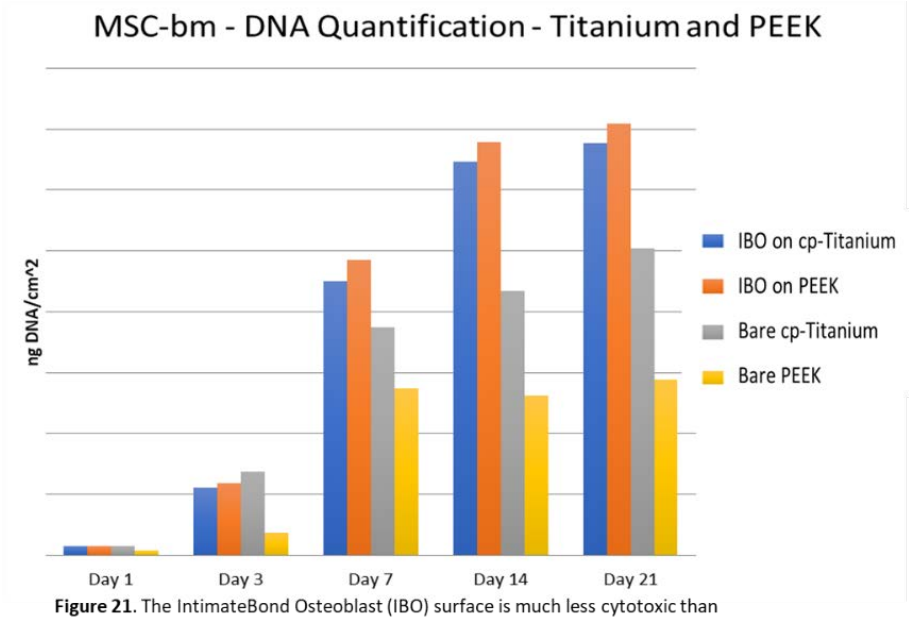


Figure 21. The IntimateBond Osteoblast (IBO) surface is much less cytotoxic than bare PEEK.

Figure 22 shows the results of two test versions of IntimateBond™ Osteoblast surface solutions (IBO-1 and IBO-2); each is radically different in technical approach and cost to manufacture. These IBO surfaces are compared to a control of bare PEEK regarding osteoblast cell viability (hFOB 1.19). The average % viability, expressed in optical density (OD), on IBO-1 and IBO-2 showed IntimateBond™ Osteoblast surfaces have:

1. a significantly higher cell viability on each day, across the board, and
2. IntimateBond surfaces can be specifically engineered at the nano- and sub-nanoscale to achieve different levels of increased osteogenic viability. Surfaces can be formulated to achieve an approximate 3.5-fold increase in viability compared to PEEK immediately upon adhesion on Day 1 with a trend that continues to show superior increased cellular activity compared to unmodified substrates over time.

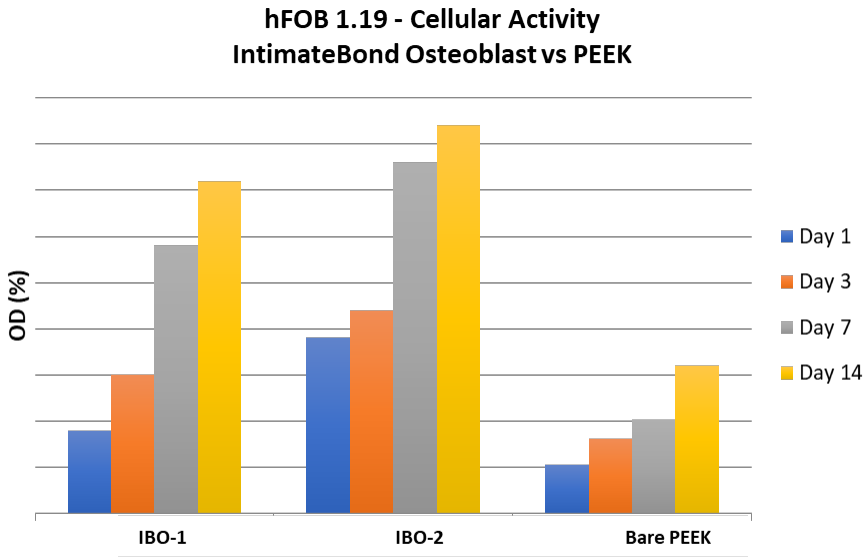


Figure 22. The IntimateBond Osteoblast (IBO) surfaces each promote significantly higher cell viability than bare PEEK.

Figure 23 illustrates the effect of IntimateBond™ Osteoblast applied to titanium testing several iterations of surface variables (IBO 1-7 and IBO A1-A4) on osteoblast (hFOB 1.19) cell count in relation to commercially pure solid titanium. The average cell count, per cm^2, on all modified substrates showed, in general, IntimateBond™ Osteoblast surfaces, even in these early tests have significantly higher cell adhesion at 4 hours and enhanced proliferative behavior through day 7 compared to the bare solid CP Titanium. For all surface modifications, a rapid increase in viable cells counted on the surface occurs first at day 3 and again at day 7. IntimateBond™ Osteoblast surfaces can be specifically manipulated via macro-/micro-/nano-type surface topography to achieve osteoblast proliferative rates ranging from approximately 2000 cells/cm^2 per day to 7000 cells/cm^2 per day.

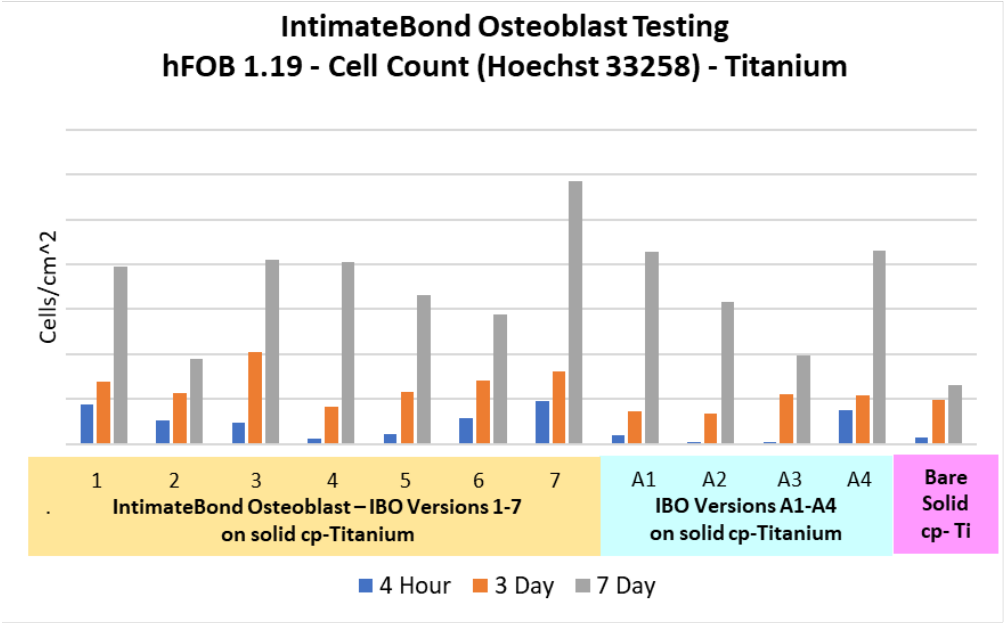


Figure 23. Early testing – Multiple versions of IntimateBond Osteoblast (IBO) surfaces applied to solid Titanium showed significantly higher cell viability than bare solid cp-Titanium.

Figure 24 compares the IntimateBond™ Osteoblast surfaces on the initial adhesion of osteoblasts (hFOB 1.1) and fibroblasts (Human Dermal Fibroblast (HDF)). Average cell count, per cm^2, on a modified metal alloy of Nickel-Titanium (Nitinol) and Cobalt-Chromium-Molybdenum (CoCrMo) versus bare metal showed IntimateBond™ Osteoblast surfaces can

1) be applied to commonly used orthopedic alloy metal surfaces to enhance adhesion of different cell types, and
2) can be applied to selectively promote and control the adhesion of one type of cell while simultaneously discouraging adhesion of a second cell type. When compared to uncoated Nitinol, fibroblasts have a significantly higher tendency (6.8-fold) to initially adhere with optimized surface compared to osteoblasts (3.3-fold). On the other hand, the IntimateBond™ Osteoblast surface can be applied to CoCrMo surfaces in order to promote the adhesion of osteoblasts while suppressing the adhesion of fibroblasts. After 4 hours, osteoblasts had adhered to modified CoCrMo with a 20-fold increase in population compared to fibroblasts.

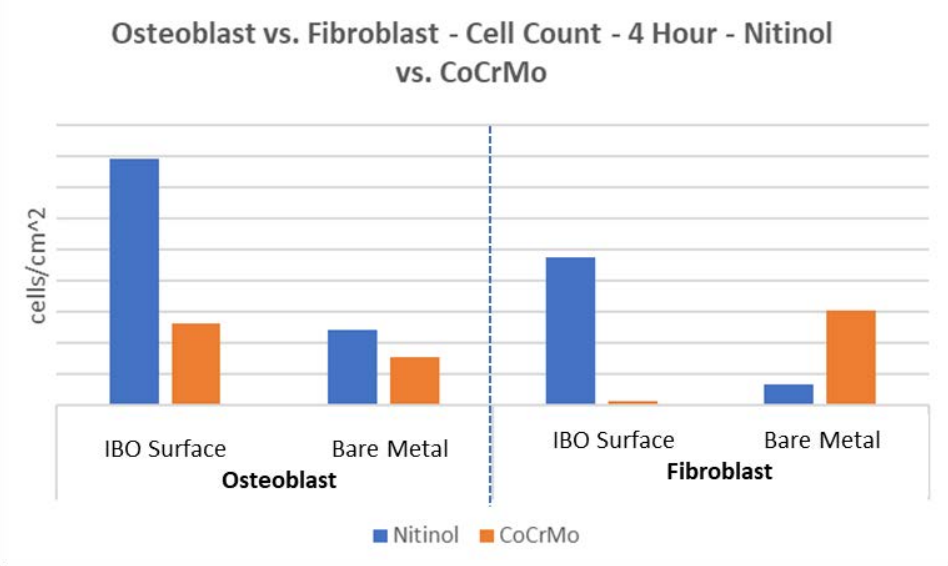


Figure 24. The IntimateBond Osteoblast surface has different effects on cells which are useful in different applications.

Figure 25 demonstrates the effect of IntimateBond™ Osteoblast competing surfaces, labeled IBO-1 and IBO-2, tested on osteoblast (hFOB 1.19) adhesion in relation to bare substrate controls: ultra-high molecular weight polyethylene (UHMWPE), polytetrafluoroethylene (PTFE), polyvinylchloride (PVC), polyethylene terephthalate (PET), 304 stainless steel (Stainless) and silicone. Osteoblasts and fibroblasts attached to all substrates modified with the Immuno-optimized IntimateBond™ Osteoblast surface, except silicone where osteoblasts did not adhere.

Both osteoblasts and fibroblasts were able to adhere to all IBO surfaces, except UHMWPE, but at a density that was significantly less than that with IntimateBond test surfaces. Both osteoblasts and fibroblasts attached to PTFE, Stainless and Silicone modified with IntimateBond IBO-2. In general, osteogenic cell adhered in greater numbers to substrates, especially on Stainless. The adhesion of fibroblasts can be selectively eliminated or severely impaired by applying the IntimateBond IBO-2 surface to materials such as PET, PVC and UHMWPE.

Average cell count, per cm^2, on modified versus control polymeric and metallic materials demonstrated that the IntimateBond™ Osteoblast surface can:

1) be applied to a variety of polymer and metal surfaces to enhance initial adhesion of different cell types, and
2) can be applied selectively to promote and control the adhesion of one type of cell while simultaneously discouraging adhesion of a second cell type.

The number of viable osteoblast cells on **immuno-optimized** UHMWPE and 304 Stainless Steel was significantly higher, 1.7-fold and 1.7-fold, respectively, compared to the unmodified surface. Fibroblastic cell growth on cp-Titanium and 304 Stainless Steel could not be selectively impaired with implementation of the **immuno-optimized** surface. Average cell count, per cm^2, on a modified versus uncoated (C) polymeric and metallic materials at day 7 demonstrated that Implant Surfaces' **immuno-optimized** surface can:

1) be applied to a variety of polymer and metal surfaces to enhance initial adhesion of different cell types and
2) can be applied selectively to promote and control the adhesion of one type of cell while simultaneously discouraging adhesion of a second cell type.

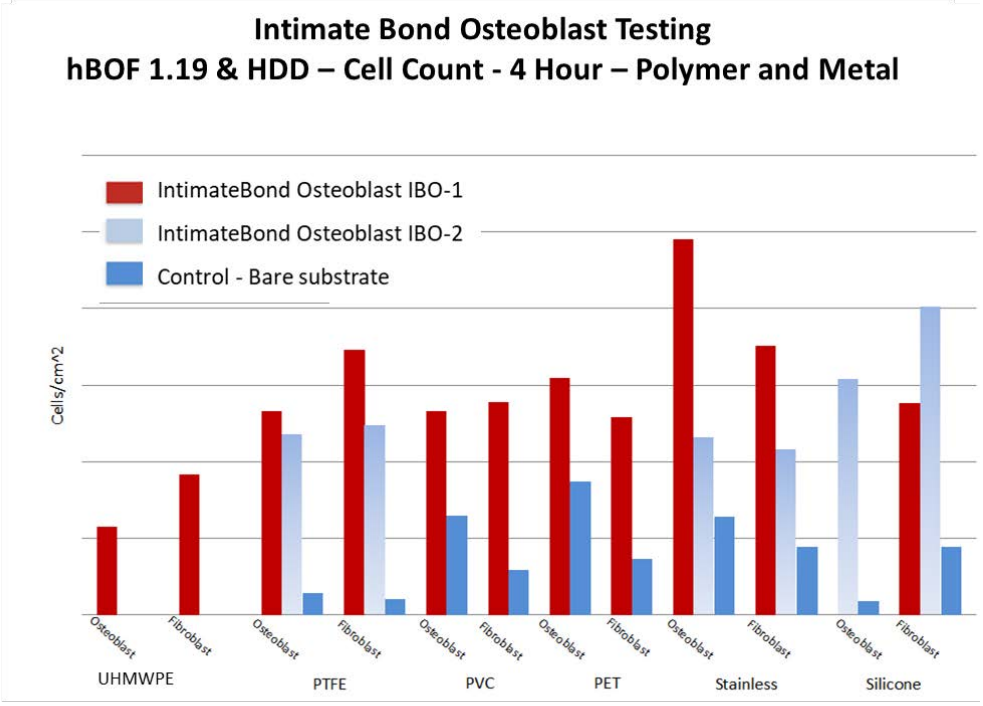


Figure 25. The competing IntimateBond Osteoblast surfaces (IBO-1 and IBO-2) applied to various polymer and metal substrates showed significantly higher cell viability than bare substrate.

IntimateBond™ Osteoblast *in vivo* Results

Following extensive *in vitro* testing, an *in vivo* study was conducted in 2009 (unpublished) to compare competing surfaces, IBO-1 and IBO-2, and the final technical approach was chosen. Later, additional *in vivo* testing by Walsh[75] published in 2018, showed in a proven sheep model, "The titanium surface coating of PEEK using the IPD [Implant Surfaces] process enhances osteoblast adhesion, spreading, proliferation and calcium deposition compared with uncoated PEEK surface."

The 2018 study evaluated the in vivo response of promoting new bone growth and bone apposition with NanoMetalene® (NM) compared with PEEK alone in a cancellous implantation site with an empty aperture. The NanoMetalene® (NM)-coated surface is SeaSpine's brand name for Implant Surfaces' exclusive IntimateBond™ Osteoblast Titanium surface, first implanted in humans in 2013. Cylindrical dowels with two open apertures were prepared as PEEK with a sub-micron layer of the titanium (NM).

Implants were placed in the cancellous bone of the medial and lateral proximal tibia and distal femur. Anteroposterior radiographs at 4 and 8 weeks are presented for Group 1, which was fully coated with NanoMetalene® (NM) on all surfaces, and Group 3, which was PEEK with no coating on any surface. NM coating is not visible on the radiographs. The implants cannot be seen because of the radiolucent nature of PEEK and normal bone anterior and posterior to the implants. Newly formed woven bone tracked along the surface of IBO titanium in the [otherwise empty] apertures [without benefit of any growth factors].

Direct bone contact to the surface to the titanium coating was observed more consistently with the NM-coated samples than PEEK, including when the NM coating was placed only inside the aperture. **Figure 26** shows magnification on the outside of the NanoMetalene® (NM)-coated surface and PEEK implants at 4 and 8 weeks. The direct contact with the NM titanium coating is shown at 4 weeks and bone contact greatly improves by 8 weeks. Bare PEEK surfaces presented the typical nonreactive fibrous tissue interface at 4 weeks with some focal bone contact at 8 weeks. Quantification of histology demonstrated direct bone contact to the NM coated surface at 4 and 8 weeks both inside the apertures and outside of the device.

Implant Surfaces' method for modulating the immune system will prevent most fibroblast issues and takes advantage of advanced immuno-compatibility to encourage the cascade to attach osteoblasts.

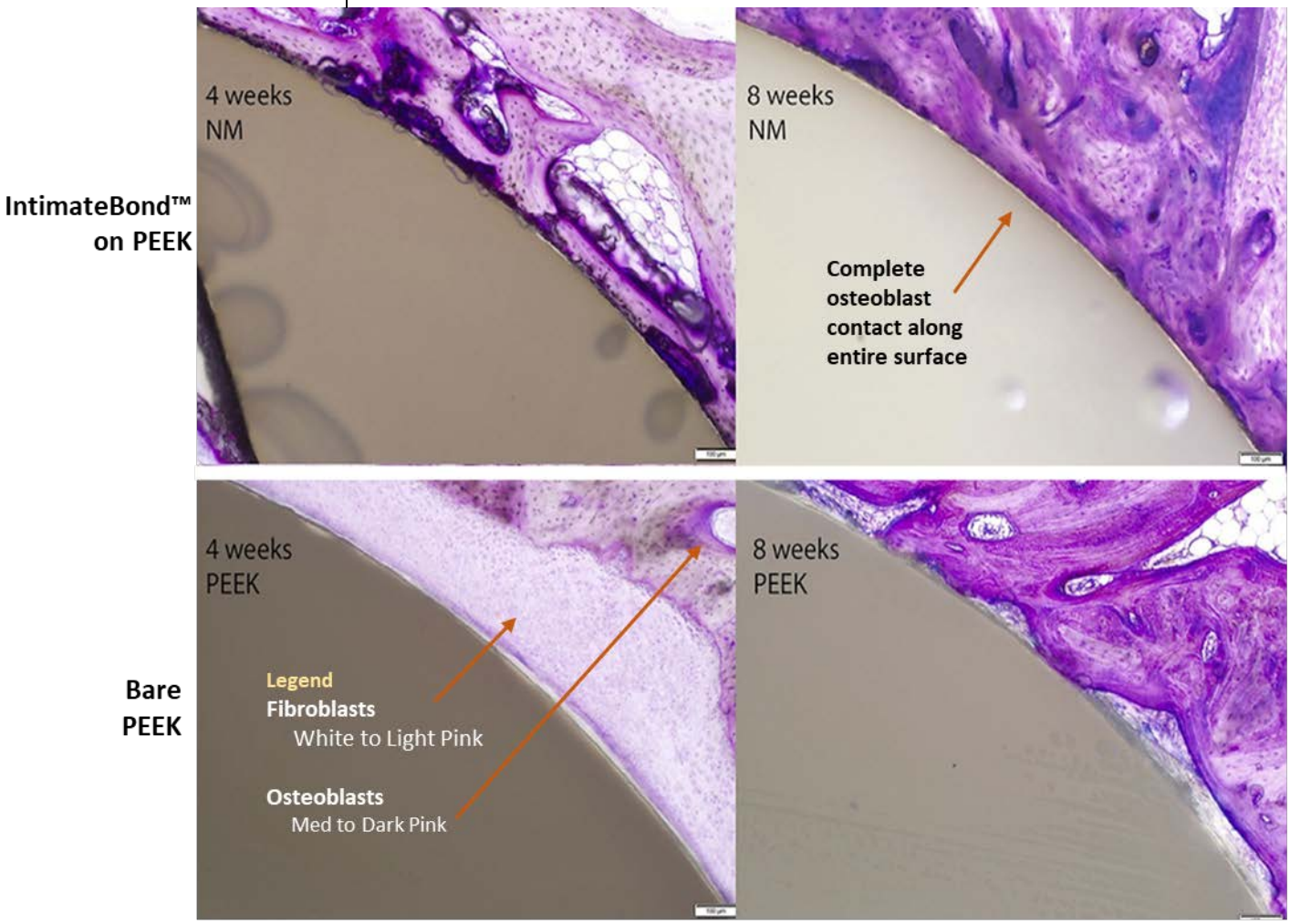


Figure 26. Typical higher magnification on the outside of the NanoMetalene® (NM)-coated surface (SeaSpine's brand name for Implant Surfaces' exclusive IntimateBond Osteoblast cp-Ti surface) and PEEK implants at 4 and 8 weeks. Direct bone contact with the titanium coating with NM at 4 weeks (upper left), which improved with time at 8 weeks (upper right). PEEK surfaces presented the typical nonreactive fibrous tissue interface at 4 weeks (white fibroblasts, lower left), with some focal bone contact at 8 weeks (lower right). (Walsh-2018)[75]

Conclusion

Described herein, **immuno-optimized** surfaces were characterized for their surface chemistry, topography and effect on protein adsorption, immunological response and effect on model cells that participate in the reconstruction of bone tissue including, polymorphonuclear cells, bone-marrow-derived mesenchymal stem cells, osteoblast cells, vascular endothelial cells and fibroblast cells compared to unmodified surfaces.

- The use of IntimateBond™ **immuno-optimized** surfaces on different types of materials with different cell types enabled interpretation of effects of Immuno-optimized surface design on bone-generating activity.
- Even though both distance and contact osteogenesis occur during reconstruction, optimizing contact osteogenesis, through an **immuno-optimized** implant surface design, is biologically significant, especially for achieving stability and strength at the interface of biomaterial with an implant.
- Though macro-/micron-scale topography have been widely used to improve interfacial fixation, combining nano-scale mechanical properties via hierarchical surface modification significantly promotes contact osteogenesis on otherwise regular or smooth surfaces.[64-65]
- Topography, at the microscale level (0.1 – 100 μm), that mimics the size and shape of extracellular matrix influences cells at the singular cell level.
    - In addition to influencing the morphological and physiochemical features of individual cells and their intracellular contacts, microtopographical structures are responsible for modulating initial cell attachment.
- Topography, at the nanoscale level (0.1 – 100 nm) impacts cells at the cellular receptor level via cytoskeletal arrangement[66-70] and is responsible for increasing the benefits of increased surface area and high surface energy required for cell-substrate affinity.
- Nano-level surface mechanical properties, on the other hand, are influential in determining the behavior (e.g., adhesion, proliferation, differentiation/gene expression) of cells on an implant and help prime the expansion of specific progenitor cells.[66,71]

Implant Surfaces' **immuno-optimized** implant surfaces can lift specification limits that are placed on surfaces that consist solely of macro-designed architecture. Considerable effort/attention has been placed on utilizing macro-/micro-scale modifications for orthopedic implant fixation.[66] A higher micro-roughness surface content has been shown to accommodate cell attachment[72-74] but fails to accelerate osteointegration[72], a key component of contact osteogenesis.

Modification of a surface with nano- and sub-nano-architecture, while preserving the macro-/micro-roughness of a surface, accommodates cell attachment while simultaneously promoting bioactivity down to the level of gene expression, a prerequisite for mineralization and *de novo* bone formation at the surface of the implant. With an appropriate macro-/micro-/nano/sub-nano surface topography, bone progenitor cells may attach, differentiate, move interdigitally and secrete *de novo* bone at the depths within a 3D-architecture. It can then be envisioned that ingrowth in this manner helps to anchor the implant to adjacent bone and extend the life of the implant.

Implant Surfaces' proprietary **immuno-optimized** surface technology, when applied to traditional macro-/micro-rough orthopedic implant surfaces, demonstrates faster and better osseointegration resulting in superior ingrowth and device fixation. **immuno-optimized** surfaces provide unlimited design and dimension capabilities.

Additionally, Implant Surfaces' surface modification methods and techniques accommodate custom surface modification products for clients that require unique improvements. Single-ordered (e.g., macro-, micro-, nano- or sub-nano-) or multi-ordered (e.g., macro-/nano- or micro-/nano-) structures in a number of shapes (e.g., spinulose, cube, increased surface area, etc.) and densities can be constructed on a number of different biomaterial surfaces (e.g., metallic, polymeric, ceramic, composite, etc.) using surface modification methods and techniques that accommodate both porous and non-porous implants.

Considering the versatility of Implant Surfaces' **immuno-optimized** surfaces, a variety of topographical structures can be applied to a surface to control factors that correlate with the extent of implant biocompatibility which in turn affects the method and degree of cellular response for tissue integration.

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